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Bosentan, a dual endothelin receptor antagonist, activates the pregnane X nuclear receptor

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Abstract

Recent clinical studies have shown that bosentan, a dual endothelin receptor antagonist, decreases the exposure to various substrates of cytochrome *P*450 (CYP) isoenzymes 2C9 and 3A4. The aim of the study was to investigate the effect of bosentan, its metabolites and glibenclamide on the activity of the pregnane X receptor, a nuclear receptor that regulates the transcription of CYP3A4. CV-1 monkey kidney cells were transiently transfected with a luciferase reporter plasmid containing three copies of the ER6 response element of CYP3A4 and the human or mouse pregnane X receptor. Subsequently, the cells were incubated with the test compounds and the activity of luciferase determined. Bosentan activated the human pregnane X receptor with an EC_{50} of 19.9 μM, whereas rifampicin had an EC_{50} value of 1.9 μM. Ro 47-8634 (4-*tert*-butyl-*N*-[6-(2-hydroxy-ethoxy)-5-(2-hydroxy-phenoxy)-2,2′-bipyrimidin-4-yl]-benzenesulfonamide), a metabolite of bosentan, and glibenclamide also activated the pregnane X receptor. The findings provide a molecular mechanism for the interactions observed between bosentan and several drugs.

Keywords: Bosentan; Glibenclamide; Pregnane X receptor; Cytochrome *P*450 enzyme induction

1. Introduction

The cytochrome *P*450 (CYP) isoenzymes are a superfamily of heme-containing proteins that are mainly located in the liver but that can also be found in the gut, brain and lungs (Flockhart and Oesterheld, 2000). They play a crucial role in the metabolism of both endogenous compounds and xenobiotics, including drugs. Some drugs induce the transcription of several genes encoding CYP isoenzymes and this may lead to important drug–drug interactions. Classical examples of CYP inducers are rifampicin and phenobarbital (Landrum Michalets, 1998). Recently, bosentan (Tracleer™) has been identified as a compound with CYP-inducing potential.

Bosentan is an orally active, non-peptidic, competitive dual endothelin receptor antagonist with high affinity for both endothelin ET_A and ET_B receptors (Clozel et al., 1994). It was the first endothelin receptor antagonist to enter clinical development. Currently, bosentan is marketed for the treatment of pulmonary arterial hypertension (Channick et al., 2001) and was investigated in chronic heart failure (Kiowski et al., 1995; Ellahham et al., 2000). Bosentan has been shown to be an inducer of different CYP isoenzymes. Drug–drug interaction studies in healthy volunteers with warfarin (Weber et al., 1999a), glibenclamide (Van Giersbergen et al., 2002) and cyclosporine A (Binet et al., 2000), and multiple-dose studies with bosentan showing autoinduction of metabolizing enzymes and increased urinary excretion of 6β-hydroxycortisol (Weber et al., 1999c) provided evidence for the induction properties of bosentan. Measurement of the urinary excretion of 6β-hydroxycortisol has been proposed as a non-invasive method to monitor induction of CYP3A4 by drugs in man (Ohnhaus and Park, 1979; Ged et al., 1989).

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The metabolism of bosentan is dependent on the CYP2C9 and 3A4 isoenzymes. Three metabolites have been identified: Ro 47-8634 (4-*tert*-butyl-*N*-[6-(2-hydroxy-ethoxy)-5-(2-hy-droxy-phenoxy)-2,2′-bipyrimidin-4-yl]-benzenesulfonamide), formed by *O*-demethylation, Ro 48-5033 (4-(2-hydroxy-1,1-dimethyl-ethyl)-*N*-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2′-bipyrimidin-4-yl]-benzenesulfonamide), formed by hydroxylation and the secondary metabolite, Ro 64-1056 (4-(2-hydroxy-1,1-dimethyl-ethyl)-*N*-[6-(2-hydroxy-ethoxy)-5-(2-hydroxy-phenoxy)-2,2′-bipyrimidin-4-yl]-benzenesulfonamide), formed by the combination of *O*-demethylation and hydroxylation (Weber et al., 1999b). It is unknown how bosentan induces CYP isoenzymes and whether the inducing potential resides with the parent compound and/or with its metabolites.

Drugs may induce metabolising enzymes in a number of ways: induction via the aryl hydrocarbon receptor; induction via the peroxisome proliferator activated receptor; induction by activation of the pregnane X receptor and/or the constitutive androstane receptor (for review see Waxman, 1999; Fuhr, 2000; Honkakoski and Negishi, 2000). The pregnane X receptor has been identified as a nuclear receptor that can be activated by pregnanes and other compounds and constitutes a novel steroid signalling pathway (Bertilsson et al., 1998; Kliewer et al., 1998; Blumberg et al., 1998). Several lines of evidence indicate that the pregnane X receptor is implicated in mediating the effects of compounds that induce CYP3A gene expression. The pregnane X receptor is abundantly expressed in the tissues, i.e., liver and gut, in which CYP3A induction occurs, it binds to response elements in CYP3A gene promotor regions and is activated by compounds that are known inducers of CYP3A (Bertilsson et al., 1998; Kliewer et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998).

The objectives of this study were to investigate the effects of bosentan and its metabolites on pregnane X receptor activation. In a drug–drug interaction study in healthy subjects, it was noted that concomitant administration of glibenclamide, a blood glucose lowering drug of the sulfonylurea class, and bosentan resulted in reduced plasma concentrations of the latter (Van Giersbergen et al., 2002). Therefore, the effects of glibenclamide alone and in combination with bosentan on pregnane X receptor activation were investigated. Furthermore, the effect of bosentan on CYP3A4 protein content in human hepatocytes was studied.

2. Materials and methods

2.1. Chemicals

Bosentan, Ro 47-8634, Ro 48-5033, and Ro 64-1056 were synthesized by F. Hoffmann-La Roche. Dulbecco's Modified Eagle's Medium (DMEM)/F-12, foetal calf and bovine serum, rifampicin, glibenclamide and 5-pregnane-3β-ol-20-one-16α-carbonitrile (PCN) were obtained from Sigma (St. Louis, MO, USA). Chlorophenol red-1β-galactopyranoside was purchased from Roche Molecular Biochemicals (Rotkreuz, Switzerland), passive lysis buffer (E194A) and luciferin (E1483) from Promega (Zürich, Switzerland), and LipofectAMINE reagent from Life Technologies (Basel, Switzerland).

2.2. Plasmids

The expression vectors for the human and mouse pregnane X receptor (pSG5-hPXR and pSG5-mPXR) were kindly provided by Dr. S.A. Kliewer (GlaxoSmithKline, Research Triangle Park, NC, USA). The expression vector for β-galactosidase and the reporter vector, CYP3A4-(ER6)3-tkluc, were kindly provided by Drs. N. Kralli (Division of Biochemistry, Biozentrum, University of Basel, Switzerland) and R.M. Evans (Gene Expression Laboratory, The Salk Institute for Biological Studies, La Jolla, CA, USA), respectively.

2.3. Transcriptional activation assays

CV-1 monkey kidney cells were maintained in DMEM/F-12 supplemented with 10% foetal bovine serum. Before experiments, CV-1 cells were plated in 96-well plates at a density of 50,000 cells per well in DMEM/F-12 medium without phenol red, supplemented with 10% charcoal-stripped foetal bovine serum. Cells were transiently transfected using LipofectAMINE reagent according to the manufacturer's instructions. Transfection assay mixtures contained 20 ng of reporter plasmid, 60 ng of β-galactosidase expression vector, and 8 ng of expression vector (human or mouse pregnane X receptor). Twenty-four hours after transfection, the medium was replaced by DMEM/F-12 without phenol red, supplemented with 10% delipidated, charcoal stripped foetal calf serum containing the compounds of interest. Cells were then incubated for an additional 24 h. After induction, cell extracts were prepared using passive lysis buffer and assayed for luciferase activity with luciferin using a Wallac Victor multilabel counter (Berthold Technologies, Regensdorf, Switzerland). β-Galactosidase activities were determined by adding 180 μl of chlorophenol red-1β-galactopyranoside substrate solution (0.5 mM chlorophenol red-1β-galactopyranoside, 20 mM 2-mercaptoethanol, 60 mM Na_2HPO_4, 45 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) to 20 μl of cell lysate. After 10 min incubation at 37 °C, absorption at 550 nm wavelength was measured using a Labsystems Multiskan RC microplate reader (Labsystems, Frankfurt am Main, Germany).

2.4. Pregnane X receptor transactivation experiments

In initial experiments, CV-1 cells transfected with the mouse or human pregnane X receptor were incubated with

bosentan, Ro 47-8634, Ro 48-5033, Ro 64-1056, glibenclamide (all at final concentrations of 1, 5 or 25 μM), rifampicin and PCN (both at a final concentration of 10 μM). In addition, concentration–response curves for bosentan and rifampicin (final concentrations 0.1–50 μM) were determined in CV-1 cells under the same conditions.

A potential synergistic interaction between bosentan and glibenclamide in activating the human pregnane X receptor was tested by co-incubating CV-1 cells with bosentan (final concentration 1 μM) and different concentrations of glibenclamide (final concentrations 0.5, 1 or 5 μM).

2.5. Data analysis

Luciferase activities were normalised against β-galactosidase values to compensate for varying transfection efficiencies. Luciferase values were then standardised against untreated control cells and expressed in fold activation. Data represent the mean ± S.D. of three independent experiments. EC_{50} concentrations (i.e., the concentration eliciting 50% of the maximal response) were calculated using GraphPad Prism version 3.02 (GraphPad Software, San Diego, CA, USA).

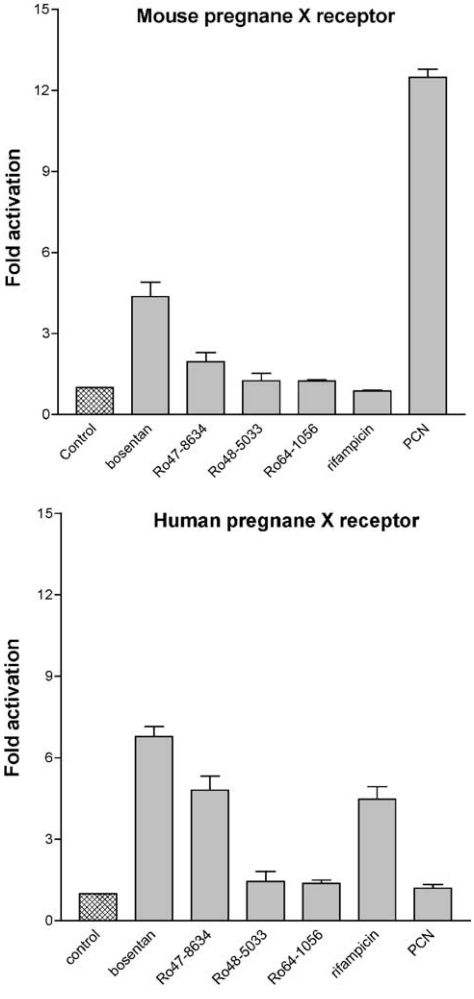

Fig. 1. Effect of bosentan and its metabolites, Ro 47-8634, Ro 48-5033, and Ro 64-1056, on the activity of the mouse (top panel) and human (bottom panel) pregnane X receptor transiently transfected in CV-1 cells. Compounds were tested at a final concentration of 25 μM and rifampicin and PCN (both at 10 μM) were included in the experiment as positive controls. Data represent means ± S.D., $n = 3$.

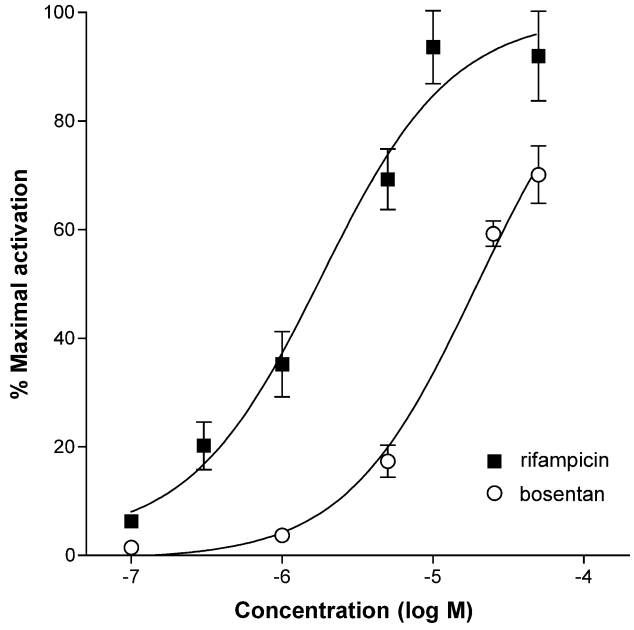

Fig. 2. Comparison of concentration–response curves of bosentan and rifampicin. CV-1 cells transiently transfected with the human pregnane X receptors were exposed to bosentan and rifampicin at concentrations ranging form 0.1 to 50 μM. Data represent means ± S.D., $n = 3$.

2.6. Experiments with human hepatocytes

Human liver tissue was obtained from 4 patients with metastatic colon cancer who required a hepatic lobectomy. The French National Ethics Committee approved the use of such tissue for scientific purposes. The preparation of hepatocytes in primary culture was performed as described previously (Pichard et al., 1990; Diaz et al., 1990). Cells were cultured in collagen-coated dishes in a medium consisting of a 1/1 mixture of Ham F12 and Williams' E media supplemented as published (Isom and Georgoff, 1984). During the first 4 h of culture, 5% foetal calf serum was added to favour attachment of the cells. Then, the medium was renewed (without serum) and subsequently every 24 h. Cultured hepatocytes were treated with bosentan (final concentrations 5 or 50 μM) or with rifampicin (final concentration 25 μM) for 96 h with renewal of treatment every 24 h when the medium was changed. CYP3A4 content was quantified by western blot analysis of microsomes prepared from treated cells as previously described using a specific CYP3A4 antibody (Diaz et al., 1990; Curi-Pedrosa et al.,

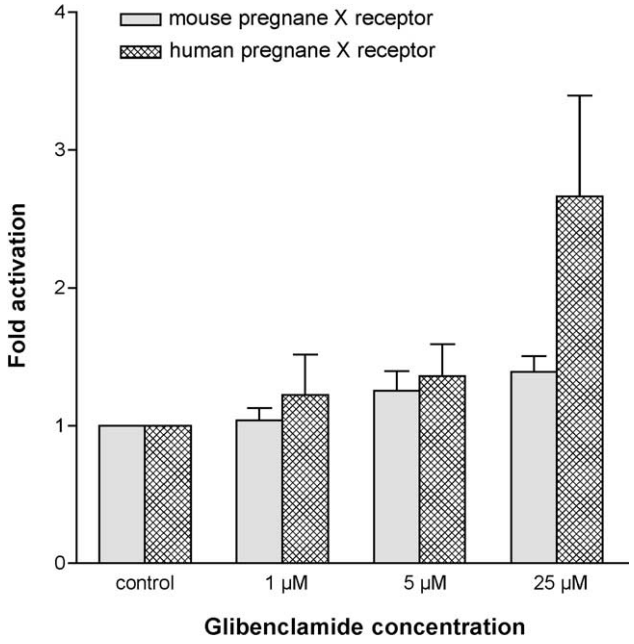

Fig. 3. Concentration-dependent effect of glibenclamide on the activity of the mouse and human pregnane X receptor transiently transfected in CV-1 cells. CV-1 cells were incubated with glibenclamide at concentrations ranging from 1 to 25 μM. Data represent means ± S.D., $n = 3$.

1994). The metabolites of bosentan were not tested in this system.

3. Results

3.1. Effect of bosentan and its metabolites on the pregnane X receptor

Bosentan at a concentration of 25 μM caused a 4.1- and 6.6-fold activation of mouse and human pregnane X receptor, respectively. At this concentration, Ro 47-8634 also activated the human pregnane X receptor (5.1-fold activation) but was less efficacious on the mouse receptor (1.8-fold activation). The effects of bosentan and Ro 47-8634, at concentrations of 1 and 5 μM were smaller than at 25 μM (data not shown). Both Ro 48-5033 and Ro 64-1056 at concentrations up to 25 μM had no effect on either the mouse or human pregnane X receptor (Fig. 1). On the human pregnane X receptor, the effect of bosentan was concentration-dependent (Fig. 2) and the EC_{50} was 19.9 ± 4.1 μM. Bosentan was less potent than rifampicin (Fig. 2), which had an EC_{50} value of 1.9 ± 0.9 μM. The positive control rifampicin activated the human pregnane X receptor but had no effect on the mouse receptor. In contrast, PCN activated the mouse but not the human pregnane X receptor (Fig. 1).

Table 1
Effects of incubation with bosentan and glibenclamide on the activation of the human pregnane X receptor

Treatment	Fold activation
Control	1.0 ± 0.0
Bosentan 1 μM	1.38 ± 0.09
Glibenclamide 0.5 μM	1.09 ± 0.06
Bosentan 1 μM + glibenclamide 0.5 μM	1.52 ± 0.22
Glibenclamide 1 μM	1.03 ± 0.08
Bosentan 1 μM + glibenclamide 1 μM	1.57 ± 0.17
Glibenclamide 5 μM	1.37 ± 0.22
Bosentan 1 μM + glibenclamide 5 μM	1.77 ± 0.18

Values are means ± S.D., $n = 3$ in each experiment.

Table 2
Increase in CYP3A4 protein content in primary human hepatocyte cultures treated with bosentan relative to the increase in CYP3A4 protein content in rifampicin-treated cultures

Treatment	Primary human hepatocyte culture (arbitrary number)			
	1	2	3	4
Rifampicin 25 μM	100	100	100	100
Bosentan 5 μM	0	0	27	15
Bosentan 50 μM	0	0	23	1.2

Values are percentage CYP3A4 protein increase relative to rifampicin-treated cultures.

3.2. Effect of glibenclamide on pregnane X receptor

Glibenclamide caused a concentration-dependent activation of the human pregnane X receptor but had little effect on the mouse receptor (Fig. 3). Concentrations higher than 25 μM were not tested and, therefore, no EC_{50} value for glibenclamide was determined. Exposure of CV-1 cells to sub-maximal concentrations of glibenclamide and bosentan resulted in a more pronounced activation of the human pregnane X receptor than with each compound alone (Table 1), although the difference was small. The effects of both compounds were additive, no synergism was detected.

3.3. Effect of bosentan in primary human hepatocytes

In all cultures tested, rifampicin induced CYP3A4 expression, whereas inducing effects of bosentan were detected in hepatocyte cultures from only two of four patients. Furthermore, the extent of induction was smaller with bosentan than with rifampicin (Table 2).

4. Discussion

Bosentan has been shown in vivo to be an inducer of CYP2C9 and CYP3A4. The mechanism by which bosentan exerts its effects on CYP gene expression is unknown. Recently, the pregnane X receptor, a nuclear receptor, has been identified as an important regulator of the expression of the CYP3A4 gene and, therefore, we investigated the effect of bosentan on the activity of this receptor.

In CV-1 cells transiently expressing the human pregnane X receptor incubation with bosentan resulted in a concen-

tration-dependent activation of this receptor as measured by reporter gene assays. The maintenance dose of bosentan in clinical practice is 125 mg twice a day (Channick et al., 2001). At this dose, the maximum plasma concentration is about 2 μM (Dingemanse et al., 2002), i.e., 10-fold lower than the EC_{50} for pregnane X receptor activation. However, in the liver, the major organ for xenobiotic detoxification via CYP isoenzymes, it is likely that the concentrations of bosentan are higher than in plasma because this compound is mainly excreted hepatically (Weber et al., 1999b). In fact, a whole body autoradiographic study in rats has shown that radioactivity levels in the liver were 30–100 times higher than in blood (Actelion Pharmaceuticals data on file). Thus, activation of the pregnane X receptor may represent the mechanism via which bosentan induces CYP3A4. This notion is further supported by the observation that in cultured primary human hepatocytes bosentan induced the expression of CYP3A4 in 2 of 4 cultures. It is unclear why only two out of four hepatocyte cultures responded to bosentan but all four to rifampicin. The response of human hepatocytes has been shown to be variable limiting their use (Smith, 2000) but the above observation may also indicate that in man, bosentan may induce CYP3A4 in some but not all patients.

In the present study, bosentan was less potent on the activation of the pregnane X receptor and in cultured primary human hepatocytes than rifampicin, a well-known and potent enzyme inducer in man and rabbits but not in mice and rats (Borcherding et al., 1992). When comparing the effects of concomitant administration of bosentan or rifampicin, given at doses used in clinical practice, on the plasma levels of drugs that are metabolized by either CYP2C9 or CYP3A4 in human subjects, it was noted that rifampicin caused greater reductions in these levels than bosentan in accordance with the above finding. For example, bosentan reduced the plasma levels of simvastatin and its active metabolite, β-hydroxy simvastatin, by 34% and 46%, respectively (Dingemanse et al., 2002), whereas rifampicin caused reductions of 87% and 93%, respectively (Kyrklund et al., 2000). Therefore, bosentan should cause fewer clinically relevant interactions with other drugs than rifampicin.

Bosentan activated both the human and mouse pregnane X receptor. In contrast, rifampicin and PCN activated either the human or the mouse pregnane X receptor, respectively, in agreement with earlier observations (Jones et al., 2000). Although the amino acid sequences of the human and mouse pregnane X receptors are remarkably different, the mutation of only four amino acids in the ligand-binding domain of the mouse receptor can change its ligand specificity to a more human-like phenotype (Watkins et al., 2001). Ro 47-8634 had a similar qualitative profile as rifampicin, whereas the other two metabolites of bosentan, Ro 48-5033 and Ro 64-1056, were inactive at the highest concentration tested. The contribution of Ro 47-8634 to the CYP-inducing properties of bosentan in man is unknown at present but is likely to be small. The concentrations of this metabolite in plasma are much lower than those of bosentan (Dingemanse et al., 2002) and also in faeces it is a minor metabolite (Weber et al., 1999b).

It is interesting to note that the metabolite Ro 48-5033 retains affinity to the endothelin receptors (Actelion Pharmaceuticals data on file) but not to the human pregnane X receptor, whereas for Ro 47-8634 the opposite was observed. A similar structure–activity relationship as for activation of the pregnane X receptor was demonstrated for inhibition of the bile salt export pump in rat canalicular liver plasma membranes (Fattinger et al., 2001). Bosentan, Ro 47-8634 and, to a smaller extent, Ro 48-5033 inhibited this pump which is important for the excretion of bile salts into bile. Some bile salts, e.g., lithocholic acid, are metabolised by CYP3A4 and can be hepatotoxic. A recently proposed mechanism to limit accumulation of bile salts is the induction of CYP3A4 via the pregnane X receptor by some of these compounds (Schuetz et al., 2001). In fact, Xie et al. (2001) have shown that the hepatotoxic effects of lithocholic acid were less pronounced in wild-type mice than in mice lacking the pregnane X receptor. However, in both strains, lithocholic acid was able to induce CYP3A4 expression. This indicates that an alternative mechanism to pregnane X receptor activation exists via which bile salts may induce CYP3A4 expression. By inhibiting the bile salt export pump, bosentan may cause accumulation of bile salts possibly leading to a secondary induction of CYP3A4. The importance of this alternative molecular mechanism to direct activation of the pregnane X receptor for the in vivo CYP3A4-inducing properties of bosentan in man is unknown.

Glibenclamide also has been shown to inhibit the bile salt export pump (Fattinger et al., 2001) and may induce CYP3A4 via secondary accumulation of bile salts. In cultured hepatocytes, glibenclamide increased CYP3A activity but not its protein expression (Pichard et al., 1990). The present study has shown that glibenclamide can activate the pregnane X receptor albeit at concentrations that are much higher than the peak plasma concentrations ($\approx$ 0.15 μM) observed in diabetic patients (Jönsson et al., 2000). This apparent discrepancy prompted us to investigate a possible synergistic effect of bosentan and glibenclamide on pregnane X receptor activity but no such effect could be demonstrated. It should be noted, however, that concentrations measured in plasma possibly do not reflect those in the liver. Therefore, induction of CYP3A4 by glibenclamide either directly by activation of the pregnane X receptor or indirectly via inhibition of the bile salt export pump may explain the decrease in plasma concentrations of bosentan observed in healthy subjects concomitantly treated with glibenclamide (Van Giersbergen et al., 2002).

Until recently, the regulation of expression of the CYP2C9 gene was poorly understood. Work of Gerbal-Chaloin et al. (2002) has shown that the CYP2C9 gene is mainly regulated by a glucocorticoid receptor but its expres-

sion may also be upregulated by the pregnane X receptor and the constitutive androstane receptor. Therefore, the possibility exist that bosentan induces CYP2C9 also by activating the pregnane X receptor. At present, is it unknown if bosentan also interacts with the constitutive androstane receptor but this is conceivable as a number of compounds appear to activate both nuclear receptors (Moore et al., 2000).

In conclusion, bosentan activates the human and mouse pregnane X receptor. These results provide a molecular mechanism for the CYP-inducing properties of bosentan in man.

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